

The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis

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ABSTRACT The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane that is commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors. Quantitative or semiquantitative methods may be used to evaluate the amount of angiogenesis and anti-angiogenesis. Thanks to the CAM system, angiogenesis could be investigated in association with normal, inflammatory and tumor tissues, and soluble factors inducing angiogenic or anti-angiogenic effects could be identified. Rabbit cornea provides an alternative *in vivo* system, but CAM appears to be easier to handle and less expensive. Moreover, CAM can be used with very few limitations.

KEY WORDS: angiogenesis, anti-angiogenesis, chorioallantoic membrane, chick embryo

Introduction

In vivo and *in vitro* techniques are available for research on the functions of endothelial cells during angiogenesis (Auerbach *et al.*, 1991). Of the *in vivo* techniques, those utilizing chick extraembryonic membranes, namely the chorioallantoic membrane (CAM) and the area vasculosa, both of which are copiously vascularized, as well as the one employing the rabbit cornea, i.e. an avascular organ, are the most reliable ones.

Histogenesis and structure of CAM

The allantois is an extraembryonic membrane, derived from the mesoderm, in which primitive blood vessels begin to take shape on day 3 of incubation. On day 4, the allantois merges with the chorion epithelium, derived from the ectoderm, to form the chorioallantois (Romanoff, 1960) (Fig. 1A,B). Primitive vessels continue to proliferate and to differentiate into an arteriovenous system until day 8 (Fig. 2A), thus originating a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment (Fig. 2B). Rapid capillary proliferation goes until day 11; thereafter, their mitotic index declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching (Ausprunk *et al.*, 1974).

In ovo utilization

Fertilized eggs staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) are placed into an incubator

as soon as embryogenesis starts and are kept under constant humidity at 37°C. At HH stage 20, a square window is opened into the shell after removing 2-3 ml of albumen so that the developing chorioallantois is detached from the shell itself and the underlying CAM vessels are disclosed (Fig. 3A,B). The opening is closed with a cellophane tape and incubation goes on until the day of the experiment.

In vitro utilization

By other methods, the CAM vascular system is displayed in greater detail, except that the embryo and the extraembryonic membranes must be transferred to a Petri dish in the early stages of development, i.e. on days 3 or 4 of incubation (Auerbach *et al.*, 1974) (Fig. 3C). There CAM develops at the top as a flat membrane, reaching the edge of the dish to provide a two-dimensional monolayer onto which grafts can be placed. Because the entire membrane can be seen, rather than just a small portion through the shell window, multiple grafts can be placed on each CAM and photographs can be taken periodically to document vascular changes over time.

Subsequently, several modifications of this method have been described (Dugan *et al.*, 1981; Dunn *et al.*, 1981; Jakobson *et al.*, 1989). Dugan *et al.* (1981) used an inert plastic container (instead

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Abbreviations used in this paper: CAM, chorioallantoic membrane; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TNF- α , tumor necrosis factor alpha; TGF- β , transforming growth factor beta; EGF, epidermal growth factor; PDGF, platelet derived growth factor

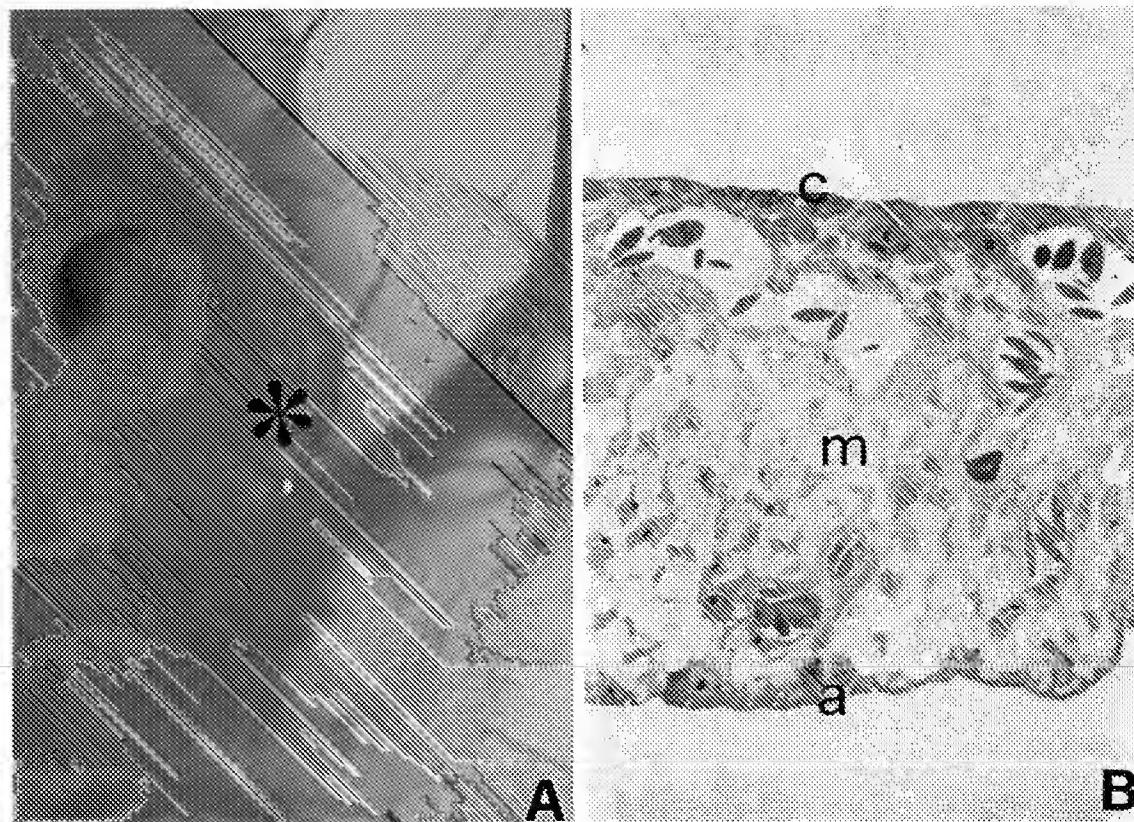


Fig. 1. Macroscopic and microscopic CAM structure. (A) Allantoic sac (*lastenaki*) at a 5-day embryo showing *in ovo* distribution pattern of allantoic vessels. (B) Histological pictures of the CAM of 8-day chick embryo showing chorion epithelium (c), intermediate mesenchyme (m) and allantoic endoderm (a). A, $\times 25$; B, $\times 160$.

of a Petri dish) equipped with a "parafilm" ring (4.5 cm inside depth) to provide support for the embryo and its membranes. Advantages include somewhat longer viability and lower costs, though these are offset by the difficulty of monitoring angiogenesis during incubation and by the fact that one cannot obtain two-dimensional photographs suitable for image analysis.

Testing substances and tissues

The test substance is soaked in inert synthetic polymers laid upon the CAM. Elvax 40 (ethylene-vinyl acetate copolymer) and hydron (a poly-2-hydroxyethyl-methacrylate polymer, HydroMed Sciences) are commonly used. The two polymers were first described and validated by Langer and Folkman (1976); both proved to be biologically inert when implanted onto the CAM and both were found to polymerize in the presence of the test substance, allowing its sustained release during the assay. However, hydron requires the test substance to be added to a solution of hydron and ethanol. When the test pellets are vacuum-dried, ethanol is removed leaving a solid pellet that contains the test substance. If the test material is not compatible with ethanol, Elvax can be used instead. Elvax is dissolved in methylene chloride before the test material is suspended/dissolved in the polymer, after which methylene chloride is removed by vacuum-drying. A more sustained release can be achieved by "sandwiching" the test substance between two Elvax layers. The polymers cause the substance to be released at constant rates (nanograms to micrograms) around-the-clock.

Alternatively, when testing a fluid substance, the latter is inoculated (20–60 µl) directly into the cavity of the allantoic vesicle,

so that its activity will develop evenly over the whole vascular area (Ribatti *et al.*, 1987).

Another method has been recently proposed by Nguyen *et al.* (1994): by this method, a collagen gel is conjugated with the testing substance and placed between two pieces of mesh (bottom layer 4x4 mm, upper layer 2x2 mm). The resulting "sandwich" is then placed upon the CAM on day 8 of incubation.

Tissues to be tested are converted under sterile conditions in minute bits (1–2 mm³) that are gently placed onto the CAM with a fine forceps on day 8–9 of incubation.

When polymers are used in combination with an angiogenic substance, a vasoproliferative response will be recognizable 72–96 h after implantation: the response takes the form of increased vessel density around the implant, with the vessels radially converging towards the centre like spokes in a wheel (Fig. 4A–D). Conversely, when polymers combined with an anti-angiogenic substance are tested, then vessels become less dense around the implant after about 72–96 h, and eventually disappear (Fig. 4E).

Lastly, when the substance is inoculated into the cavity of the allantoic vesicle, then the angiogenic or anti-angiogenic response affects the CAM vessels as a whole.

Evaluating the vasoproliferative response by semiquantitative methods

Several semiquantitative methods are used to evaluate the extent of the vasoproliferative response. One method considers changes in the distribution and density of CAM vessels next to the implant which are evaluated *in vivo* by means of a

stereomicroscope at regular intervals following the graft procedure. The score is 0 when no changes can be seen; it is +1 when few neovessels converge towards the implant, and +2 when a considerable change in the number and distribution of the converging neovessels is observed (Knighton *et al.*, 1977).

By another method, the vasoproliferative response is graded as a vascular index derived from photographic reconstructions. All converging neovessels contained inside a 1 mm-in-diameter ring superimposed upon the CAM are counted: the ring is drawn around the implant in such a way that it will form an angle of less than 45° with respect to a straight line drawn from the implant's centre. Vessels branching dichotomically outside the ring are counted as 2, while those branching inside the ring area counted as 1 (Dusseau *et al.*, 1986).

A third method measures the degree of vasoproliferative response, as evaluated under the stereomicroscope, by an arbitrary 0-to-6 scale. Zero describes a condition of the vascular network that shows no change from the time of grafting; +1 marks a slight increase in the vessel density associated to occasional changes in the course of vessels converging toward the implant; +2, +3, +4 and +5 indicate a progressive increase in vessel density associated with more pronounced changes in their course, while a +6 score also highlights strong hyperemia. A coefficient describing the degree of angiogenesis can also be derived from the ratio of the calculated value to the highest attainable value. Therefore, the coefficient's lowest value is 0 and the highest is 1 (Folkman and Shing, 1976).

Strick *et al.* (1991) calculate the length of the vessels and express it in terms of index density, i.e. the vessel density relative to a fractional image area of the vasculature. By using the above mentioned method of Nguyen *et al.* (1994), they express the vasoproliferative response after 72–216 h as a percentage of the squares in the upper mesh occupied by neovessels. The effect of the inhibitory substances (placed on the bottom mesh) is quantified by calculating the inhibition of the vasoproliferative response induced by an angiogenic factor such as, for example, the basic fibroblast growth factor (bFGF).

Evaluating the vasoproliferative response by quantitative methods

Quantitative evaluation of vessel density can be obtained by applying morphometric and planimetric methods to histologic

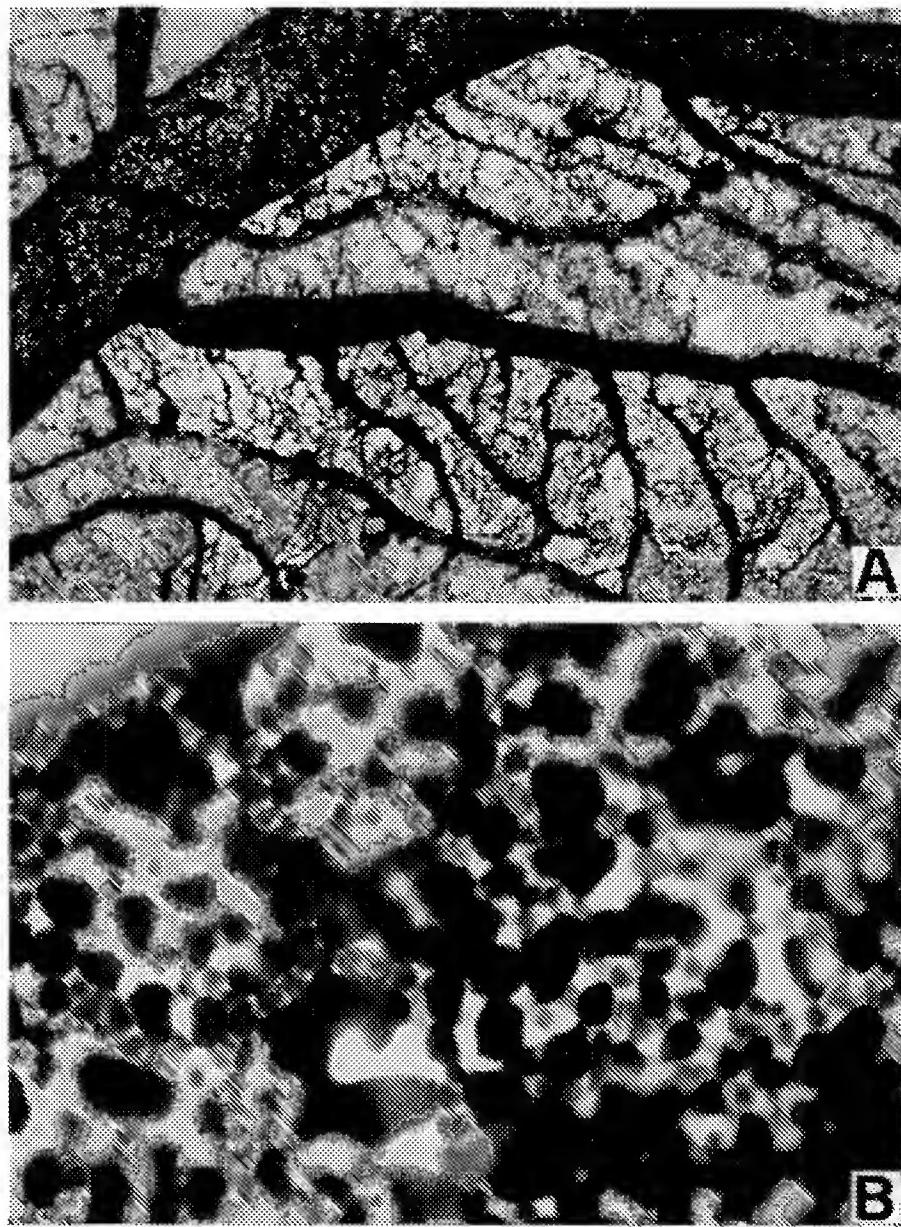


Fig. 2. Macroscopic features of CAM vasculature. (A) Image of the CAM arteriovenous system after an *in vivo* intravascular injection of India ink. (B) Image of the CAM superficial capillary network after an *in vivo* intravascular injection of fluorescein-sucroseyanate-labeled dextran. A, $\times 100$; B, $\times 400$.

observations of CAM specimens fixed at regular intervals after implantation (Fig. 5). The number of vessels is evaluated as the total number of vessels present in 6 randomly chosen microscopic fields ($\times 200$). Vessel density is evaluated by a planimetric method (Elias and Hyde, 1983) which utilizes a square reticule (with 144 points of intersection of 12 lines per side) placed in the eyepiece of a photomicroscope. Six randomly chosen fields per section are observed and the total number of intersection points occupied by transversally sectioned vessels is counted. Vessel density equals this total number, expressed as the percent value of all intersection points. Evaluation of the number and density of vessels should be made by two independent observers and processed statistically.

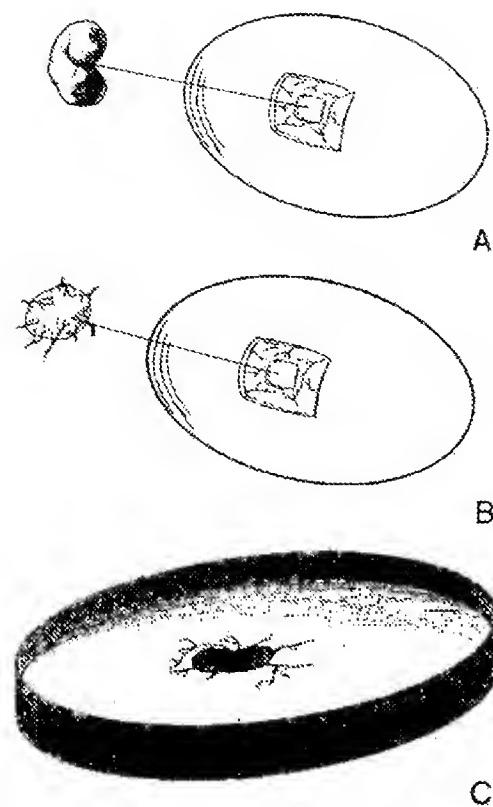


Fig. 3. Different CAM uses. (A,B) Drawings showing the *in ovo* use of CAM vasculature for embryonic (A) or tumoral (B) tissue grafts. (C) Drawing showing the *in vitro* use of CAM vasculature utilizing a Petri dish.

Another method measures the ^{3}H -thymidine incorporated into the CAM. The amount incorporated into the DNA is expressed as a percentage of total ^{3}H -thymidine present in the CAM homogenate, regardless of the CAM weight and full recovery of applied radioactivity (Thompson *et al.*, 1982; Siplawinski *et al.*, 1988). Automatic image analysis has also been applied (Jakob and Voss, 1984; Tanara *et al.*, 1986).

The limitations of CAM

CAM finds its main limitation in non-specific inflammatory reactions which may develop from the implant and in turn induce a secondary vaso-proliferative response, eventually making it difficult to quantify the primary response that is being investigated (Jakob *et al.*, 1978; Spanel-Borowski *et al.*, 1988); in this respect, a histologic study of CAM sections would help to detect the possible presence of a perivascular inflammatory infiltrate together with a hyperplastic reaction, if any, of the chorion epithelium. In an extensive series of experiments by Jakob *et al.* (1978), a variety of carrier vehicles alone (Millipore filters, fiber glass discs, gelatine and viscose sponges, discs of filter paper, agarose and polyacrylamide gels), as well as natural egg components (egg shell membrane, coagulated albumin and coagulated yolk) produced a number of inflammatory reactions.

However, the possibilities of causing a non-specific inflammatory reaction are much lower when the implant is made very

early in CAM development, since at that time the host's immune system is relatively immature. Earlier lymphoid cells deriving from the yolk sac and spleen are usually recognizable in the thymus on day 8 and in the Fabricius bursa on day 11 (Leene *et al.*, 1973).

There are three more drawbacks to the CAM assay. First, the test material is placed on pre-existing vessels, so neovascularization and the re-arrangement of vessels can hardly be distinguished from each other (Knighton *et al.*, 1991). Second, timing of the CAM angiogenic response is essential. Many studies determine angiogenesis after 24 h, a time at which there is no angiogenesis, but only vasodilatation. It would be worthwhile to point out that measurements of vessel density are really measurements of visible vessel density, and that the distinction between vasodilatation and neovascularization is not easy to make. To circumvent this drawback it is useful to utilize sequential photography to document new vessel formation. Third, often polymers do not adhere to the CAM surface. To circumvent this drawback, Folkman (1984) hydrated the testing material with 5 μl H_2O on sterile coverslide glass, which is turned over and placed onto the CAM on day 9-10 of incubation. The possible angiogenic response is evaluated 48 h later.

A similar approach has been tried by Wilting *et al.* (1991, 1992): they used culture coverslide glasses (Thermanox) 4-5 mm-diameter, on which 5 μl of several angiogenic factors were placed. Glasses were turned over and placed onto the CAM on day 9 of incubation, and the angiogenic response was evaluated 96 h later.

Saline solutions should be avoided, in that the hyperosmotic effect of crystal salts may damage the chorion epithelium and induce fibroblast proliferation (Wilting *et al.*, 1991). This implies that the substance be used at concentrations of picograms to milligrams: higher concentrations would indeed cause the hyperosmotic effect (Wilting *et al.*, 1992).

Extracts from a number of normal cells (Barnhill *et al.*, 1984; Leibovich *et al.*, 1987; Auerbach *et al.*, 1991; Ribatti *et al.*, 1991) and tissues (Ausprung *et al.*, 1977) have been found to stimulate vessel growth when implanted onto CAM.

Ausprung *et al.* (1977) compared the behavior of tumor grafts (see below) to grafts of normal adult and embryo tissues. In tumor tissue, pre-existing blood vessels in the tumor graft disintegrated within 24 h after implantation, and revascularization occurred by penetration of proliferating host vessels into the tumor tissue. By contrast, pre-existing vessels did not disintegrate in the embryo graft and anastomosed to the host vessels with almost no neovascularization. In adult tissues, pre-existing graft vessels disintegrated (although this process was slower than in tumor vessels) and did not stimulate capillary proliferation in the host. Lastly, tumor vessels did not reattach to those of the host.

These studies suggest that only tumor grafts are capable of stimulating formation of new blood vessels in the host, thus gaining their blood supply.

Angiogenesis induced by chronic inflammatory tissues

Chronic inflammation is accompanied by neovascularization as an obligatory means to sustain it. For example, in arthritis new

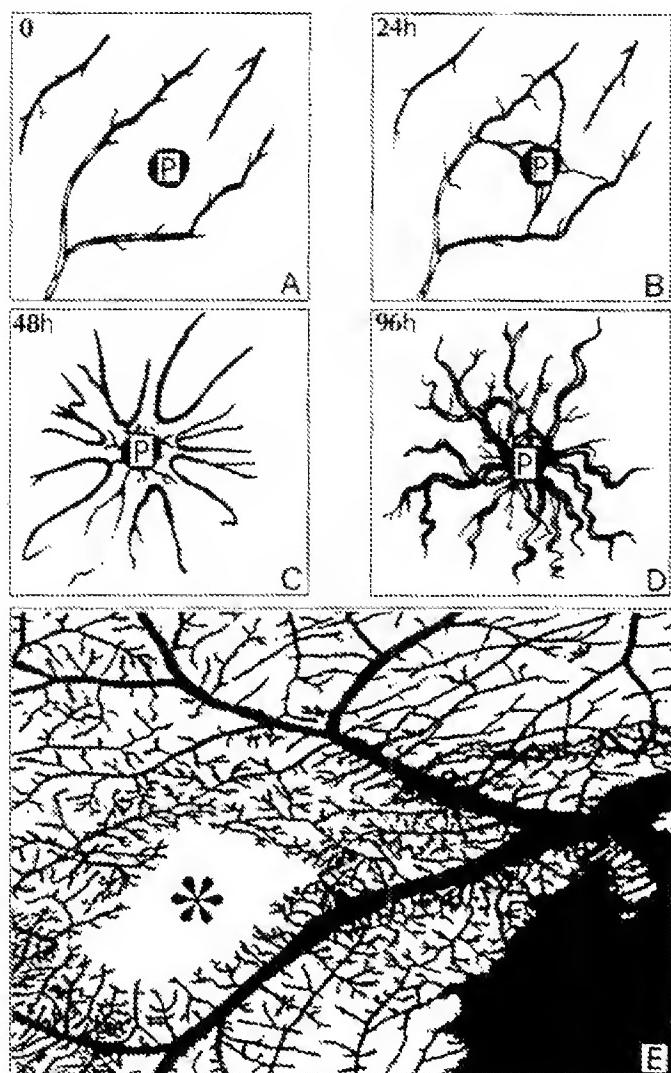


Fig. 4. The angiogenic and the antiangiogenic responses of CAM vasculature (A-D). Drawings of the time course of CAM response to a polymer (*P*) soaked with an angiogenic substance. (Modified from Folkman and Shing, 1979). (E) Drawing of an avascular zone (asterisk) in a CAM exposed to an antiangiogenic substance.

capillary blood vessels invade the joint and destroy cartilage; in diabetes, new capillaries in the retina invade the vitreous and cause blindness.

Hypoxia has been identified as a key regulator of angiogenesis and a potential source of angiogenic factors, such as the vascular endothelial growth factor (VEGF).

The angiogenic activity of several chronic inflammatory processes, such as rheumatoid arthritis (Brown *et al.*, 1980), diabetic retinopathy (Hill *et al.*, 1989) and of hypoxia (Dusseau and Hutchins, 1988) has been tested onto CAM.

Tumor angiogenesis

Tumor angiogenesis is a prerequisite for tumor growth, while the growth of new blood vessels appears to be "switched on" at

TABLE 1

STUDIES DEMONSTRATING THE ANGIOGENIC ACTIVITY OF VARIOUS SUBSTANCES ONTO CAM

Author(s)	Substance
Frisser <i>et al.</i> , 1979	Adenosindiphosphate (ADP)
McAuslan <i>et al.</i> , 1983	Copper
Forni and Auerbach, 1983	Prostaglandin E ₂ (PGE ₂)
Thompson <i>et al.</i> , 1982, 1985	Fibrin degradation products
McAuslan <i>et al.</i> , 1983	Heparin*
Ribatti <i>et al.</i> , 1987	Heparin*
Vleet <i>et al.</i> , 1988	Degradation products of hyaluronic acid
Morris <i>et al.</i> , 1988	Phorbol esters

*When absorbed on methylcellulose discs and applied onto the CAM, heparin exerts an antiangiogenic effect (Jainobina and Hahnemann, 1991).

some point after a tumor becomes established (Folkman, 1986; Vacca *et al.*, 1993). Metastasis, too, is angiogenesis-dependent: correlations between microvessel density in tumor and future metastases have been reported by various investigators (Gasparini, 1994; Vacca *et al.*, 1994).

Knighton *et al.* (1977) first described the onset (from day 6 to 16) of tumor vascularization in the CAM having used implants of fresh Walker 256 carcinoma. Chick capillaries proliferated in the vicinity of the tumor graft about 24 h after implantation, but capillary sprouts did not penetrate the graft until approximately 72 h later. During the avascular interval, tumor diameter did not exceed 1 mm, but grew rapidly during the first 24 h following capillary penetration.

The angiogenic activity of other malignant neoplasms, such as melanoma (Auerbach *et al.*, 1976; Klagsbrun *et al.*, 1976), meningioma (Klagsbrun *et al.*, 1976; Szwarcz *et al.*, 1988), glioblastoma (Klagsbrun *et al.*, 1976), lymphomas (Mostafa *et al.*, 1980; Ribatti *et al.*, 1990), endometrial carcinoma (Szwarcz

TABLE 2

STUDIES DEMONSTRATING THE ANGIOGENIC ACTIVITY OF SEVERAL GROWTH FACTORS ONTO CAM

Author(s)	Growth factor
Esch <i>et al.</i> , 1988	Basic fibroblast growth factor (bFGF)
Witing <i>et al.</i> , 1991	
Olivo <i>et al.</i> , 1992	
Ribatti <i>et al.</i> , 1992	
Fett <i>et al.</i> , 1985	Angiogenin
Witing <i>et al.</i> , 1991	
Olivo <i>et al.</i> , 1992	
Leibovich <i>et al.</i> , 1987	Tumor necrosis factor alpha (TNF- α)
Olivo <i>et al.</i> , 1992	
Roberts and Spiro, 1989	Transforming growth factor beta (TGF- β)
Stewart <i>et al.</i> , 1989	Epidermal growth factor (EGF)
Witing <i>et al.</i> , 1992	Platelet derived growth factor (PDGF)
Witing <i>et al.</i> , 1992, 1993	Vascular endothelial growth factor (VEGF)

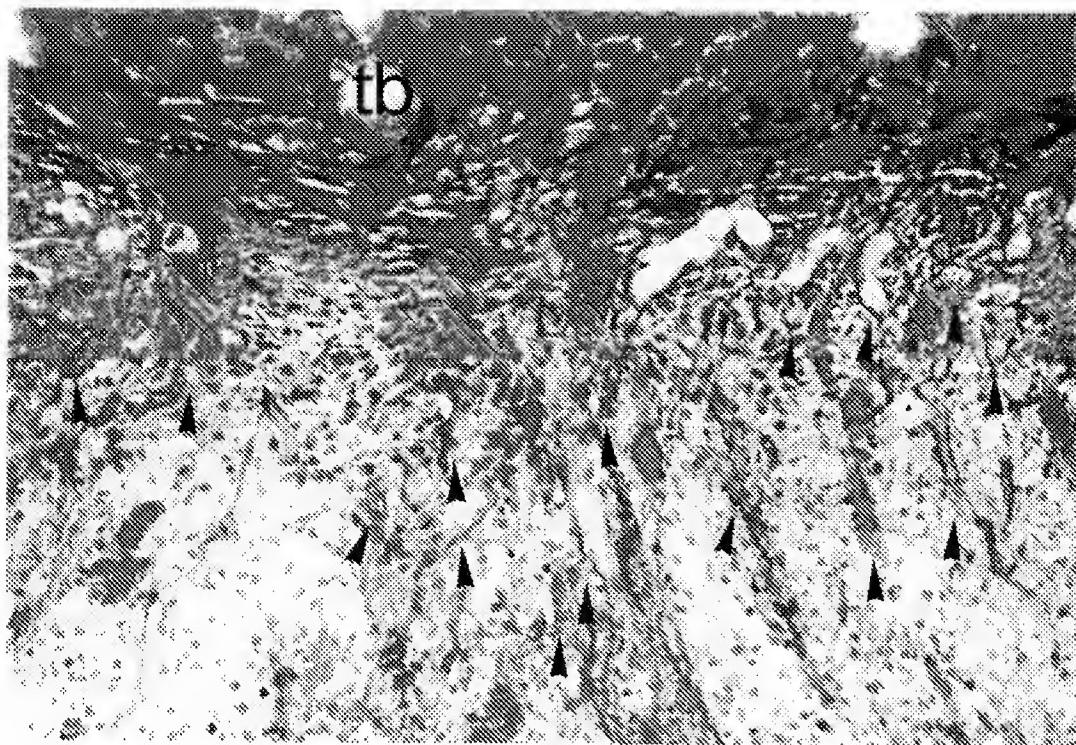


Fig. 5. The vasoproliferative response of CAM vasculature to a tumor implant. Histological picture showing the positive angiogenic response in the CAM's intermediate mesenchyme on incubation day 12, 90 h after implantation of a lymph node biopsy involved by a high grade B-cell non-Hodgkin's lymphoma. New blood vessels (arrowheads) converge toward the tumor base (tb).

et al., 1988; Ribatti et al., 1996a) and head squamous cell carcinoma (Petrizzelli et al., 1993), has been tested onto the CAM.

Testing angiogenic substances

Several substances cooperate in making up the natural microenvironment in which angiogenesis takes place. For example, "the microenvironment of a solid tumor *in vivo* can be defined

as the space between the basement membrane of the microvessels and the surface of the neoplastic cells. It is characterized by two components – the stroma and the interstitial fluid" (Gullino, 1995).

Studies demonstrating the angiogenic activity of various substances upon the CAM are summarized in Table 1. Generally, all the substances in the list are regular constituents of both the stroma and the interstitial fluid.

Testing angiogenic factors

Reports on the angiogenic activity of several growth factors on CAM are summed up in Table 2.

Among such factors, VEGF specifically induces blood vessel growth (Wilting et al., 1992, 1993). Concerning the pivotal angiogenic role of another growth factor, the basic fibroblast growth factor (bFGF), Wilting et al. (1993) stated that "...bFGF strongly increases the proliferation rate evenly throughout the stroma of the CAM, which means that is mainly fibrocytes and only to a minor degree endothelial cells that respond to the factor". Recently we have clearly demonstrated that endogenous bFGF has a rate-limiting role in the vascularization of the CAM (Ribatti et al., 1995).

Testing anti-angiogenic substances

Angiogenesis appears to depend on the balance of several stimulating and inhibiting factors. Inhibition of blood vessel growth, i.e. anti-angiogenesis, or else stimulation of angiogenesis-inhibiting factors, would seem to provide a strategy for preventing both the growth of tumors and other angiogenesis-dependent diseases (Auerbach and Auerbach, 1994).

TABLE 3

STUDIES DEMONSTRATING THE ANTI-ANGIOGENIC ACTIVITY OF VARIOUS SUBSTANCES ONTO CAM

Authors	Substance
Eisenstein et al., 1978	Chondroityna derived est-β-hor
Taylor and Folkman, 1982	Prostaglandins
Folkman et al., 1983	Heparan or heparin fragments + cortisone
Drum et al., 1985	Heparin+11-hydroxysterone or 17-hydroxyprogesterone
Ingram and Folkman, 1988	Analogs of proteins
Mengelkoch et al., 1988	Inhibition of basement membrane biosynthesis
Folkman et al., 1989	Beta cyclooxygenase-2 adenosulfate (COX-2) + angiostatic steroids
Oikawa et al., 1989	Butyric acid
Misra et al., 1990	Plasminogen activator
Ishikawa et al., 1990	Vitamin D ₃ analogues
Ingraham et al., 1990	Argenoxazine
Wichterling et al., 1991	Gomatoestatin
Wilkins et al., 1991	Steroid+angiostatic steroids
O'Reilly et al., 1994	Angiostatin
Saukkonen et al., 1994	Heparinase
Ribatti et al., 1996a	Interferon alpha 2a

Studies demonstrating the anti-angiogenic activity of several substances on the CAM are summarized in Table 3.

Concluding remarks

CAM is widely utilized as an *in vivo* system to study angiogenesis and anti-angiogenesis. Although rabbit cornea is used just as often as an *in vivo* system, CAM offers the advantage of being simpler to use and less expensive. Moreover, unlike the rabbit cornea, the CAM system allows several assays to be carried out simultaneously in the same embryo, especially when the *in vitro* approach is used instead. On the other hand, there are only very few restrictions to using CAM, essentially due to: a) non-specific inflammatory reactions that may develop with an attending secondary stimulation of angiogenesis and, b) pre-existing vessels may be present which make it hard to distinguish the extent of angiogenesis and anti-angiogenesis.

An important step forward in using the CAM system, which we ourselves are working on, consists in isolating the endothelial cells from the CAM vessels and culturing them to test angiogenic or anti-angiogenic factors. A parallel research on these factors upon the CAM will enable us to understand to what extent the periendothelial microenvironment provided by CAM as a whole is capable of affecting angiogenesis or anti-angiogenesis.

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References

- AUERBACH, R., AUERBACH, W. and POLAKOWSKI, I. (1991). Assays for angiogenesis: a review. *Pharmacol. Rev.* 43: 1-11.
- AUERBACH, R., KUBAI, L. and SHIN, Y.A. (1976). Angiogenesis induction by tumors, embryonic tissues and lymphocytes. *Cancer Res.* 36: 3505-3549.
- AUERBACH, R., KUBAI, L., KNIGHTON, D. and FOLKMAN, J. (1974). A simple procedure for the long-term cultivation of chicken embryos. *Dev. Biol.* 41: 381-394.
- AUERBACH, W. and AUERBACH, R. (1994). Angiogenesis inhibition, a review. *Pharmacol. Ther.* 63: 265-311.
- AUSPRUNK, D., KNIGHTON, D. and FOLKMAN, J. (1974). Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. *Dev. Biol.* 38: 237-249.
- AUSPRUNK, D., KNIGHTON, D. and FOLKMAN, J. (1977). Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. *Am. J. Pathol.* 87: 607-618.
- BARNHILL, B.L., PARKINSON, E.K. and RYAN, T.J. (1984). Supernatants from cultured human epidermal keratinocytes stimulate angiogenesis. *Brit. J. Dermatol.* 110: 273-281.
- BROWN, P.A., WEISS, J.B. and TOMLINSON, I.W. (1980). Angiogenic factor from synovial fluid resembling that from tumors. *Lancet* i: 682-685.
- CRUM, R., SZABO, S. and FOLKMAN, J. (1985). A new class of steroid inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 230: 1375-1378.
- DUGAN, J.D. Jr., LAWTON, M.T., GRASER, B. and BREM, H. (1981). A new technique for explantation and *in vitro* cultivation of chicken embryos. *Anat. Rec.* 200: 125-128.
- DUNN, B., FITZHARRIS, T.P. and BARNETT, B.D. (1981). Effects of varying chamber construction and embryos pre-incubation age on survival and growth of chick embryos in shell-less culture. *Anat. Rec.* 200: 33-43.
- DUSSEAU, J.W. and HUTCHINS, P.M. (1986). Hypoxia-induced angiogenesis in the chick chorioallantoic membrane: a role for adenosine. *Respir. Physiol.* 61: 33-44.
- DUSSEAU, J.W., HUTCHINS, P.M. and MALBABA, D.B. (1986). Stimulation of angiogenesis by adenosine on the chick chorioallantoic membrane. *Circ. Res.* 58: 162-170.
- EISENSTEIN, R., KUETTNER, K.E., NEOPOLITAN, C., SOBEL, L.W. and SORGENTE, N. (1975). The resistance of certain vessels to invasion. III. Cartilage extract inhibits the growth of fibroblasts and endothelial cells in culture. *Am. J. Pathol.* 87: 337-348.
- ELIAS, H. and HYDE, G.M. (1988). Stereological measurements of isotropic structures. In *A Guide to Practical Stereology* (Eds. H. Elias and D.M. Hyde), Karger, Basel, pp. 23-44.
- ESCH, F., BAIRD, A., LINQ, N., YENON, R., HILL, F., DENORBY, L., KLEPPNER, R., GOSPODAROWICZ, D., BOHLEN, P. and GUILLEMOT, B. (1988). Primary structure of bovine pituitary bFGF and comparison with the amino-terminal sequence of bovine aPGF. *Proc. Natl. Acad. Sci. USA* 85: 8597-8601.
- FETT, J.W., STRYDOM, D.J., LOSS, A.R., ALDERMAN, E.M., BETTMUN, J.L., MORDAN, J.F. and VALLEE, B.L. (1989). Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry* 24: 6480-6489.
- FOLKMAN, J. (1994). Angiogenesis. In *Biology of Endothelial Cell* (Ed. E.A. Janes), Martin Dunitz Publishers, Boston, pp. 412-428.
- FOLKMAN, J. (1995). Tumor angiogenesis. In *The Molecular Basis of Cancer* (Eds. J. Mendelsohn, P.M. Howley, M.A. Israel and L.A. Liotta), WB Saunders, New York, pp. 208-232.
- FOLKMAN, J. and ODEMAN, B. (1975). Relation of vascular proliferation to tumor growth. *Mit. Rev. Exp. Pathol.* 18: 287-298.
- FOLKMAN, J., LANGER, R., LINHARDT, R.C., HAUDENSCHILD, C. and TAYLOR, S. (1988). Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of laminin. *Science* 241: 719-726.
- FOLKMAN, J., WEISS, P.B., JULLIAC, M.M., LI, W.W. and EWING, R. (1989). Control of angiogenesis with synthetic heparin substitutes. *Science* 243: 1490-1493.
- FORM, D. and AUERBACH, R. (1983). PGF₂ and angiogenesis. *Proc. Soc. Exp. Biol. Med.* 177: 214-218.
- FRASER, R.A., ELLIS, M. and STALKER, A.L. (1979). Experimental angiogenesis in the CAM. In *Current Advances in Basic and Clinical Microcirculation Research* (Ed. D.H. Lewis), Karger, Basel, pp. 25-26.
- GASPARINI, G. (1984). Quantification of intratumoral vascularization predicts metastasis in human invasive solid tumours. *Oncol. Rep.* 1: 7-12.
- GULLINO, P.M. (1985). Prostaglandins and gangliosides of tumor microenvironment: their role in angiogenesis. *Acta Oncol.* 24: 439-441.
- HAMEURÄGER, V. and HAMILTON, H.L. (1953). A series of normal stages in development of the chick embryo. *J. Morphol.* 90: 45-92.
- HILL, C.R., KISSIL, R.D., WEISS, J.B. and GARNER, A. (1983). Angiogenic factor in vitreous from diabetic retinopathy. *Experientia* 39: 583-585.
- INGBER, D. and FOLKMAN, J. (1988). Inhibition of angiogenesis through modulation of collagen metabolism. *Lab. Invest.* 59: 44-51.
- INGBER, D., FUJITA, T., KISHIMOTO, S., KANAMARU, T., SUKO, N., BREM, H. and FOLKMAN, J. (1990). Angiostatin: synthetic analogues of tumaglobin which inhibit angiogenesis and suppress tumor growth. *Nature* 345: 555-557.
- JAKOB, W. and VOSS, K. (1984). Utilization of image analysis for the quantification of vascular responses in the chick chorioallantoic membrane. *Exp. Pathol.* 26: 89-93.
- JAKOB, W., JENTZSCHE, R.D., MANERSBERGER, B. and HEIDER, G. (1978). The chick chorioallantoic membrane as bioassay for angiogenesis factors: reactions induced by carrier materials. *Exp. Pathol.* 15: 241-249.

- JAKOBSON, A.M. and HAHNENBERGER, R. (1991). Antiangiogenic effect of heparin and other sulfated glycosaminoglycans in the chick embryo chorioallantoic membrane. *Pharmacol. Toxicol.* 69: 122-126.
- JAKOBSON, A.M., HAHNENBERGER, R. and MAGNUSSON, A. (1989). A simple method for shell-less cultivation of chick embryos. *Pharmacol. Toxicol.* 64: 193-195.
- KLAGSBRUN, M., KNIGHTON, D. and FOLKMAN, J. (1978). Tumor angiogenesis activity in cells grown in tissue culture. *Cancer Res.* 38: 110-114.
- KNIGHTON, D., AJSPERLINK, D., TAPPER, D. and FOLKMAN, J. (1977). Avascular and vascular phases of tumor growth in the chick embryo. *Brit. J. Cancer* 35: 347-366.
- KNIGHTON, D., FIEGEL, V.D. and PHILLIPS, G.D. (1991). The assay for angiogenesis. In *Clinical and Experimental Approaches to Dermal and Epidermal Repair: Normal and Chronic Wounds*. Wiley-Liss Inc., New York, pp. 291-299.
- LANGER, R. and FOLKMAN, J. (1978). Polymers for the sustained release of proteins and other macromolecules. *Nature* 263: 797-800.
- LEENE, W., DUYZINGA, M.J.M. and VON STEEG, C. (1973). Lymphoid stem cell identification in the developing thymus and bursa of Fabricius of the chick. *Z Zellforsch.* 139: 521-533.
- LEIBOVICH, S., POLVERINI, P.J., SHEPARD, H.M., WISEMAN, D.M., SHIVELY, V. and NUSIER, N. (1987). Macrophage-induced angiogenesis is mediated by TNF-alpha. *Nature* 329: 630-632.
- MAIONE, T.E., GRAY, S.G., PETRO, J., HUNT, A.J., DONNER, A.L., BAUER, S.I., CARSON, H.F. and SHARPE, R.J. (1989). Inhibition of angiogenesis by recombinant human platelet factor 4 and related peptides. *Science* 247: 77-79.
- MARAGOUUDAKIS, M.E., SARMONIKA, M. and PANOUTSCACOPULOU, M. (1988). Inhibition of basement membrane biosynthesis prevents angiogenesis. *J. Pharmacol. Exp. Ther.* 244: 729-733.
- MCASLAN, B.R., REILLY, W.G., HANNAN, G.N. and GOUE, G.A. (1983). Angiogenic factors and their assay. Activity of formylmethionyl leucyl phenylalanine, adenosine diphosphate, heparin, copper and bovine endothelium stimulating factor. *Microvasc. Res.* 26: 333-338.
- MORRIS, P.B., HIDAT, T., BLACKSHEAR, P.J., KLINTWORTH, G.K. and SWAIN, J.L. (1988). Turner promoting phorbol ester induced angiogenesis *in vivo*. *Am. J. Physiol.* 254: C318-322.
- MOSTAFARI, L.K., JONES, D.B. and WRIGHT, D.H. (1980). Mechanisms of the induction of angiogenesis by human neoplastic lymphoid tissues: studies on the chorioallantoic membrane (CAM) of the chick embryo. *J. Pathol.* 132: 191-205.
- NGUYEN, M., SHING, Y. and FOLKMAN, J. (1994). Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. *Microvasc. Res.* 47: 31-36.
- O'REILLY, M.S., HOLMGREN, L., SHING, Y., CHEN, C., ROSENTHAL, R.A., MOSES, M., LANE, W.S., CAO, Y., SAGE, E.H. and FOLKMAN, J. (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79: 315-326.
- OIKAWA, T., HIROTANI, K., NAKAMURA, O., SHUDO, K., HIRAGUN, A. and IWAGUCHI, T. (1989). A highly potent antiangiogenic activity of retinoids. *Cancer Lett.* 48: 157-162.
- OIKAWA, T., HIROTANI, K., OGASAWARA, H., KATAYAMA, T., NAKAMURA, O., IWAGUCHI, T. and HIRAGUN, A. (1990). Inhibition of angiogenesis by vitamin D₃ analogues. *Eur. J. Pharmacol.* 178: 247-250.
- OLIVO, M., BHARDWAJ, R., SCHULZE-OSTHOF, K., SORG, C., JURGEN-JACOB, H. and FLAMME, J. (1992). A comparative study on the effects of tumor necrosis factor sigma (TNF- σ), human angiogenic factor (h-AF) and basic fibroblast growth factor (bFGF) on the chorioallantoic membrane of the chick embryo. *Anat. Rec.* 234: 105-115.
- PETRUZZELLI, G.J., SNYDERMAN, C.H., JOHNSON, J.T. and MYERS, E.N. (1990). Angiogenesis induced by head and neck squamous cell carcinoma xenografts in the chick embryo chorioallantoic membrane. *Ann. Otol. Rhinol. Laryngol.* 102: 216-221.
- RIBATTI, D., LORIA, M.P. and TURSI, A. (1991). Lymphocyte-induced angiogenesis: a morphometric study in the chick embryo chorioallantoic membrane. *Acta Anat.* 142: 334-338.
- RIBATTI, D., RONCALI, L., NICO, B. and BERTOSSI, M. (1987). Effects of exogenous heparin on the vasculogenesis of the chorioallantoic membrane. *Acta Anat.* 130: 257-263.
- RIBATTI, D., URBINATI, C., NICO, B., RUSNATI, M., RONCALI, L. and FRESTA, M. (1995). Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Dev. Biol.* 170: 39-49.
- RIBATTI, D., VACCA, A., BERTOSSI, M., DE BENEDICTIS, G., RONCALI, L. and DAMMACCO, F. (1990). Angiogenesis induced by B-cell non-Hodgkin's lymphomas. Lack of correlation with tumor malignancy and immunoregulatory phenotype. *Anticancer Res.* 10: 401-406.
- RIBATTI, D., VACCA, A., GASPARINI, G., LOVERRO, G., DIVAGNO, G., URLAHO, M., LOTESORIERE, C., RONCALI, L. and SELVAGGI, G. (1996a). Endometrial hypoplasia and endometrial carcinoma induce a vaso-proliferative response in the chick embryo chorioallantoic membrane. *Int. J. Oncol.* 8: 1149-1153.
- RIBATTI, D., VACCA, A., URLAHO, M., RIA, M., RONCALI, L. and DAMMACCO, F. (1996b). Human recombinant interferon alpha-2 inhibits angiogenesis of chick area vasculosa in shell-less culture. *Int. J. Microcirc.* 16: 185-189.
- ROBERTS, A.B. and SPORN, M.B. (1989). Regulation of endothelial cell growth, architecture and matrix synthesis by TGF-beta. *Am. J. Respir. Dis.* 140: 1128-1138.
- ROMANOFF, A.L. (1980). The extraembryonic membranes. In *The Avian Embryo: Structural and Functional Development*. MacMillan, New York, pp. 1039-1141.
- SASISEKHARAN, R., MOSES, M.A., NUGENT, M.A., COONEY, C.L. and LANGER, R. (1994). Heparinase inhibits nonvascularization. *Proc. Natl. Acad. Sci. USA* 91: 1524-1528.
- SPANEL-BOROWSKI, K., SCHNAPPER, U. and HEYMER, B. (1988). The chick chorioallantoic membrane assay in the assessment of angiogenic factors. *BioMed. Res.* 8: 253-260.
- SPLAWINSKI, J., MICHNA, M., PALCZAK, R., KONTUREK, S. and SPLAWINSKI, B. (1988). Angiogenesis: quantitative assessment by the chick chorioallantoic membrane assay. *Methods Find. Exp. Clin. Pharmacol.* 10: 221-226.
- STEWART, R., NELSON, J. and WILSON, D.J. (1988). Epidermal growth factor promotes chick embryonic angiogenesis. *Cell Biol. Int. Rep.* 13: 967-965.
- STRICK, D.M., WAYCASTER, R.L., MONTANI, J., GAY, W.J. and ADAIR, T.H. (1991). Morphometric measurements of the chorioallantoic membrane vascularization: effects of hypoxia and hyperoxia. *Am. J. Physiol.* 261: H1365-1389.
- TANARA, N.G., SAKAMOTO, N., TOHGO, A., NISHIYAMA, Y. and OGAWA, H. (1986). Inhibitory effects of anti-angiogenesis agents on nonvascularization and growth of the chorioallantoic membrane (CAM): The possibility of a new CAM assay for angiogenesis inhibition. *Exp. Pathol.* 30: 143-150.
- TAYLOR, S. and FOLKMAN, J. (1982). Prostamycin is an inhibitor of angiogenesis. *Nature* 297: 307-312.
- THOMPSON, W., CAMPBELL, R. and EVANS, T. (1985). Fibrin degradation and angiogenesis: quantitative analysis of the angiogenic responses in the chick chorioallantoic membrane. *J. Pathol.* 145: 27-37.
- THOMPSON, W., SMITH, E.B., STIRK, C.M., MARSHALL, F.I., STOUT, A.J. and KOCHCHAR, A. (1982). Angiogenesis activity of fibrin degradation products is located in fibrin fragment E. *J. Pathol.* 169: 47-57.
- VACCA, A., RIBATTI, D., RONCALI, L. and DAMMACCO, F. (1995). Angiogenesis in B cell lymphoproliferative diseases: Biological and clinical studies. *Leuk. Lymphoma* 20: 27-38.
- VACCA, A., RIBATTI, D., RONCALI, L., RAINERI, G., SERIO, G., SILVESTRIS, F. and DAMMACCO, F. (1994). Bone marrow angiogenesis and progression in multiple myeloma. *Brit. J. Haematol.* 87: 503-508.
- WEST, D.C., HAMPSON, I.N., ARNOLD, F. and KUMAR, S. (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science* 238: 1324-1326.
- WILKS, J.W., SCOTT, P.A., VRBA, L.K. and COCUZZA, J.B. (1991). Inhibition of angiogenesis with combination treatments of angiostatic steroids and suramin. *Int. J. Radiat. Oncol.* 29: 73-77.
- WILTING, J., CHRIST, B. and BÖKELOH, M. (1991). A modified chorioallantoic membrane (CAM) assay for qualitative and quantitative study of growth factors. Studies on the effects of canakin, PBS, angiogenin and bFGF. *Anat. Embryol.* 182: 259-271.

- WILTING, J., CHRIST, B. and WEICH, H.A. (1992). The effects of growth factors on the day 13 chorioallantoic membrane (CAM): a study of VEGF₁₆₄ and PDGF-BB. *Anat. Embryol.* 186: 251-257.
- WILTING, J., CHRIST, B., BOKELOH, M. and WEICH, H.A. (1993). *In vivo* effects of vascular endothelial growth factor on the chicken chorioallantoic membrane. *Cell Tissue Res.* 274: 163-172.
- WOLTERING, E.A., BABRIE, R. and O'DOORISIO, T.M. (1991). Somatostatin inhibits angiogenesis in the chick chorioallantoic membrane. *J. Surg. Res.* 50: 245-251.

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